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(57) Abstract

The invention refers to the production of recombinant gene products from cultures of the yeast *Zygosaccharomyces bailii* strains transformed with expression vectors bearing the gene coding for said proteins.

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"PRODUCTION OF HETEROLOGOUS PROTEINS FROM ZYGOSACCHAROMYCES BAILII"

Field of the Invention

The invention refers to the production of recombinant gene products from cultures of the yeast Zygosaccharomyces bailii strains transformed with expression vectors bearing the gene coding for said proteins.

Background of the Invention

Recombinant DNA technologies (genetic, protein and metabolic engineering) allow the production of a wide range of peptides and proteins in naturally-non producing cells. The introduction of a heterologous as well as of a homologous gene, along with controlling sequences, in the selected host could lead to large accumulation of useful products: for example, proteins, enzymes, hormones or antigens. The availability of significant amounts of proteins is often a highly desirable goal. For example, in the pharmaceutical field, pre-clinical and clinical trials often require substantial amounts of potentially interesting recombinant proteins. Some recombinant products are already available on the market (such as growth hormone, Tissue Plasminogen Activator, hepatitis B virus vaccine, interferons, erythropoietin), and many more are currently in the last phase of clinical trials. Production of recombinant (heterologous as well as homologous) proteins has also applications in other industrial sectors. Some recombinant products are used in food (β-galactosidase, chymosin, amylases, glucoamylase, amyloglucosidase) as well as in the textile and paper (proteases, amylases, cellulases, lipases, catalases, etc...) industries. Recombinant enzymes are useful as detergents (proteases, lipases and surfactants), and their characteristics of stereo-specificity are exploited in a wide number of bioconversions, yielding the desired chiral compound. A promising field is the application of recombinant enzymes for the development of biosensors.

The potential applications of the biosensor technology range from the human health to the environmental monitoring and to the control of industrial bioprocesses. Finally, a very interesting class of heterologous expressed genes are those giving new metabolic abilities to the host cells, allowing the use of non-conventional substrates (whey, phenols, starch, lignin, cellulose). On the other hand, metabolic engineering could increase the production of fine chemical metabolites, such as organic acids (i.e., lactic acid), amino acids (i.e., glutamic acid), vitamins, solvents (i.e., ethanol, 1-2 propanediol, butanol).

The first requisite for a successful process based on engineered cells for the production of recombinant gene products concerns the choice of the host and of the expression vector. The choice must consider different factors such as product complexity, host characteristics and production level of the desired protein. From a chronological point of view, the firsts hosts used for the production of heterologous proteins were prokaryotics: Escherichia coli and Bacillus subtilis; later on, also eukaryotic host cells were used, particularly Saccharomyces cerevisiae.

The yeast S. cerevisiae, commonly considered as a safe organism, has been used for centuries in food processes. Moreover, it is a well-known microorganism, its genome is completely sequenced and its physiology and biochemistry have been studied for a long time. This yeast is able to perform some post-translational modifications of the heterologous product, which often are important for retaining of the biological activity; such post-translational modifications cannot usually be obtained using a prokaryotic host. Finally, it is possible to drive the secretion of the desired product directly in the growth medium, thus improving the large-scale recovery and purification of a correctly folded, homogeneous product. Along the years, S. cerevisiae has been developed as host for the production of both heterologous and homologous gene products with applications in all the most important

fields of a modern society (i.e., health care, pharmaceuticals, environment, agriculture, food, chemistry) (see the reviews: Romanos M.A., Scorer C.A. and Clare J.J. (1992) Yeast 8:423-488; Sudbery P. (1996) Curr.Op.Biotechnol. 7:517-524; Lin Cereghino G.P. and Cregg J.P. (1999) Curr. Op. Biotechnol. 10:422-427).

Independently of the source of the host cells, exploitations of the rDNA techniques for the production of recombinant gene products require specific attentions. The coding sequence of the gene of interest must be compatible with the chosen host. The starting codon must be unequivocally recognised; codons coding for the aminoacids of the heterologous protein must be complemented by anticodons of the host's tRNA and, preferably, should correspond to the most representative tRNAs in the tRNAs pool of the said host. Moreover, if present, signals for post-translational modifications must be the same as, or compatible with, those of the host cell. The recombinant gene, having the above characteristics, must be placed under the control of sequence(s) regulating transcription and translation in the host cell (i.e., the expression cassette). The promoter and the terminator sequences must be carefully chosen, since they directly affect the expression levels. Other sequences can determine the fate of the recombinant protein, sorting it in the vacuole, into the nucleus, into the mitochondria or along the secretive pathways. Also the stability of the mRNA and its affinity to the translation machinery are affected by the nucleotide sequence. Once the expression cassette has been constructed, it has to be inserted into an expression vector and introduced into the recipient host cell. To this aim, several solutions are available.

The expression cassette can directly be inserted in the genome of the host, by means of recombination mechanisms, either homologous (between two identical sequences, it needs the knowledge of the target sequence) or heterologous (random along the genome, cannot be controlled). A selective marker is usually required. Auxotrophic markers complement a nutrient

request, allowing the growth of the recombinant cells on non-supplemented medium. Dominant markers are genes conferring the resistance to some toxic compounds, so that only cells bearing such a marker can grow on selective media.

Alternatively, it is possible to use episomal vectors (plasmids). Such vectors are DNA fragments able to autoreplicate into the host cells. There are different kinds of episomal vectors, depending on the host. For the well known host S. cerevisiae, the following episomal vectors are generally used (Rose A.B and Broach J.R. (1990) Methods Enzymol 85:234-279; Schneider J.C. and Guarente L. (1991) Methods Enzymol 194:373-388; Romanos M.A. et al., supra; Fukuhara H. (1995) FEMS Microbiol. Letters 131:1-9)

- 2μ-like plasmid: the heterologous gene is inserted in a vector bearing all or part of the sequences from the native 2μ plasmid of S. cerevisiae. The whole plasmids are extremely stable also without a selective pressure.
- ARS plasmid: it is a quite unstable vector, based on the characteristic of an ARS endogenous sequence, which is able to promote the DNA replication at the time of chromosomal replication.
- Centromeric plasmid: essentially this is an ARS vector that is stabilised by the addition of a centromeric sequence (CEN); these sequences drive a correct partition of the vectors during mitosis. They are very stable vectors, but are retained at a low copy number (1-2/cell).
- Linear plasmid: double-strand DNA or RNA sequences, are quite common in yeasts, and theoretically they could be used in strains lacking 2μ-like plasmids. To date, no significant data are available for such a use.
- Minichromosome: based on very long DNA fragments, very stable for the presence of telomers, in addition to the above-described ARS and CEN sequences, used for basic research purposes. Potentially they can be used to clone a large cluster of genes (typically: a complete heterologous metabolic pathway).

From the comparison of the expression levels of a large set of recombinant proteins obtained in several host cells, it is apparent that the ideal host cell is not yet available. In fact, each species has some drawbacks that should be carefully evaluated, as they are only partially overcome with a good strategy of production. These drawbacks justifie the research of new host cells, in which the negative traits are absent or attenuated.

Bacterial hosts have been used for the first productions of heterologous proteins, typically the well-known E. coli. Since heterologous products for food and pharmaceutical applications must be free from any toxic or dangerous compounds, bacterial cells do not represent the ideal host for applications in the above cited sectors. In fact, E. coli produces some toxic or potentially toxic metabolites, which must be removed with careful purification protocols. Moreover, yields of heterologous products are often lowered by the formation of large insoluble aggregates (inclusion bodies). Further, strong proteolytic activities are detrimental to the production of the heterologous proteins. S. cerevisiae offers many advantages for the production of recombinant gene products; unfortunately, this host is unable to utilise some very cheap carbon sources like starch and whey. Furthermore, this host produces high amounts of ethanol when grown in the presence of relatively high sugar concentrations; the ethanol production (determined by the expression of the Crabtree effect) can be overcome with a careful (but not economic) monitoring of the fermentative conditions. The high ethanol production lowers biomass yields and, consequently, that of the heterologous gene product. Moreover, this yeast has a secretive apparatus not suitable for the very high production levels required for industrial purposes. Finally, the secreted proteins are often «hyperglycosylated» when compared to the natural product, and therefore it is hard to obtain the production of a protein identical to the original one.

Recently, expression of recombinant gene products has been obtained in

some «non-conventional yeasts»: Hansenula polymorpha, Pichia pastoris, Kluyveromyces lactis and Yarrowia lipolytica (Buckholz R.G. and Gleeson M.A.G. (1991) Bio/Technology 9:1067-1072; Fleer R. (1992) Curr. Op. Biotechnol. 3:486-496; Gellissen G. and Hollenberg C.P. (1997) Gene 190: 87-97; Muller S., Sandal T., Kamp-Hansen P. and Dalboge H. (1998) Yeast 14: 1267-1283; Lin Cereghino G.P. and Cregg J.P., supra). Said yeasts display some interesting attitudes when compared to S. cerevisiae, such as better (sometimes) expression levels, or favourable growth characteristics, namely high efficiency of growth on low-cost or wasted substrates or ability to grow under much severe culture conditions. (Sudbery P.E., (1994) Yeast 10: 1707-1726; Romanos M.A. et al., supra; Lin Cereghino G.P. and Cregg J.P., supra).

Another «non-conventional» yeast seems to offer many advantages when compared to S. cerevisiae host cells: Zygosaccharomyces bailii. This yeast displays an exceptional resistance to several stresses. For this reason, it is one of the main economically relevant spoilage yeasts. In fact, this yeast can grow in media with low water availability, high hydrostatic pressure (Palou E., Lopez-Malo A., Barbosa-Canovas G.V., Welti-Chanes J., Davidson P.M., Swanson B.G. (1998) J. Food Prot. 61:1657-60) and (relatively to S. cerevisiae) high temperatures (Makdesi A.K. and Beuchat L.R. (1996) Int. J. Food Microbiol. 33:169-81). In addition, it tolerates high sugar concentrations. Another remarkable characteristic is its very good tolerance to acid environments, as it grows at pH values as low as 2 and with high partial CO_2 pressures. Further, this yeast can survive to high preservative concentrations, such as 600 mg/l of benzoic acid (Makdesi et al., supra) or to sorbic acid (Cole M.B., Keenan M.H. (1986) Yeast. 2:93-100). However, physiology studies and molecular genetic tools for the genus Zygosaccharomyces are very poor: at 28th December, 1999 only 11 genetic sequences from Z. bailii and 38 from Z. rouxii were available at Internet site GenBank (http://www.ncbi.nlm.nih.gov/) and only a fraction of them code for

proteins. Six different endogenous plasmids have been isolated from the genus Zygosaccharomyces: pSR1, from Z. rouxii, pSB2 from Z. bailii, pSM1 from Z. fermentati, pSB1, pSB3 and pSB4 from Z. bisporus. They are structurally and functionally related, but they do not display sequence homology among them or with the endogenous plasmid of S. cerevisiae (2μ), so that they usually are not maintained in different species. Plasmid pSR1 (6251 bp), the endogenous plasmid of Z. rouxii, is the most studied in this genus. Its structure resembles to the 2μ plasmid of S. cerevisiae, and displays a pair of inverted repeat sequences between 2 unique sequences, bearing 3 genes (R, recombinase; P and S, stability) and the sequence Z, a-cis acting locus involved in the maintaining of the plasmid. Each of the repeated sequences contains an ARS, which is also recognised by S. cerevisiae (Araki H., Jearnpipatkul A., Tatsumi H., Sakurai T., Ushio K., Muta T. and Oshima Y. (1985) J. Mol. Biol. 182:191-203). Since the sequences recognised by the recombinases are not completely overlapping (Araki H. and Oshima Y. (1989) J. Mol. Biol. 207:757-69), 2μ plasmid cannot replicate in Z. rouxii. Proteins P, R e S are characteristic too, and cannot complement between Z. rouxii e S. cerevisiae (Araki et al. (1985) supra).

Contrary to the plasmid replication origin, ARS1 chromosomal replication origin from S. cerevisiae is recognised from Z. rouxii; a centromeric plasmid with this sequence could be stably maintained (Araki H., Awane K., Irie K., Kaisho Y., Naito A. and Oshima Y. (1983) Mol.Gen. Genet. 238:120-8).

The molecular mechanisms for plasmid replication and repartition are not transferable among the Zygosaccharomyces yeast strains: this is the case of pSR1 and pSB3 (Utatsu I., Utsunomiya A. and Toh-e A. (1986) J. Gen. Microbiol. 132:1359-65). This fact is not surprising, as sequences of all isolated plasmids are very different, except for a certain homology between pSB1 and pSB4. Usually, S. cerevisiae is the less restrictive yeast, as it can

recognise ARS sequences from other sources; on the other hand, no one of the Zygosaccharomyces strains tested so far was able to replicate a plasmid bearing ARS sequences from S. cerevisiae 2μ natural plasmid.

Little attempts were made to apply the genetic knowledge of the yeast Z. rouxii for the production of heterologous proteins. The only example concerns the expression of alkaline protease from Aspergillus oryzae. The expression system is based upon the endogenous pSR1 plasmid, and the use of the endogenous promoter GAPDH; geneticin (G418) resistance is the dominant marker (Ogawa Y., Tatsumi H., Murakami S., Ishida Y., Murakami K., Masaki A., Kawabe H., Arimura H., Nakano E., Motai H. et al., (1990) Agric. & Biol. Chem. 54:2521-9).

The genetic knowledge about Z. bailii is even lower. The endogenous plasmid pSB2 (5415 bp) shows some analogies with pSR1 (Toh-e A., Araki H., Utatsu I. and Oshima Y. (1984) J. Gen. Microbiol. 130 :2527-34 ; Utatsu I., Sakamoto S., Imura T. and Toh-e A. (1987) J. Bacteriol. 169:5537-45). In addition, some linear double strand RNA plasmids have been described. (Radler F., Herzberger S., Schonig I. and Schwarz P. (1993) J. Gen. Microbiol. 139:495-500). Very recently, some information appeared about the development of genetic tools for Z. bailii. A genomic bank of the yeast has been obtained (Rodrigues F., Zeeman A. M., Sousa M. J., Steensma H. Y., Corte-Real M. and Leao C. (1999) In: Proceedings of the XIX International Conference on Yeast Genetic and Molecular Biology, Curr. Genet. 35:462); the disruption of URA3 gene has been also described (Mollapour M. and Piper P.W. (1999) In: Proceedings of the XIX International Conference on Yeast Genetic and Molecular Biology, Curr. Genet. 35:452). However, nothing has to date been published about homologous or heterologous protein expression obtained from this yeast.

Since Z. bailii yeasts can grow in very restrictive cultural conditions (pH, ionic strength, temperature, sugar and acids concentrations), they are potentially interesting from an industrial point of view. In fact, the features

described above greatly simplify many fermentation procedures. For example there is no need for strict and sophisticated control of process parameters and of medium composition. Moreover, the ability to grow at higher temperature facilitates the heat control, one of the primary problems arising during high density, large-scale fermentations. Finally, the fermentation in restrictive conditions prevents any contamination problems, thus reducing the need for expensive sterilisation steps. All those elements, together with a high specific productivity, are essential to decree the economic success of an industrial production of heterologous protein.

In view of the above considerations, it is clear the importance of the development of genetic expression system(s) for Zygosaccharomyces bailii strains, together with a fast and reliable transformation protocol for the production of recombinant proteins (i.e., heterologous and/or homologous) from such a host.

Summary of the Invention

According to a first embodiment, this invention provides Zygosaccharomyces bailii strains transformed with at least one copy of a recombinant DNA gene functionally linked to promoter sequences allowing the expression of said gene in this yeast.

The invention also provides an expression vector which allows the introduction and replication of a recombinant (rDNA) gene (homologous or heterologous) functionally linked to a promoter sequence for the production of the recombinant product.

According to a further embodiment, the invention provides a process for the production of heterologous proteins by culturing the above described engineered yeast strains in a fermentation medium and by recovering the recombinant gene product from said growth medium.

Description of the Invention

It has been found that the expression of heterologous proteins can be

obtained by modified Zygosaccharomyces bailii yeasts.

More particularly, it has been found that expression vectors useful for the introduction of genetic material into the yeast Z. bailii and for the subsequent production and recovery of recombinant gene products can be developed starting from the ARS sequences of the yeast S. cerevisiae. It has been found that such sequences, and in particular the sequence ARS1 (Tschumper G. and Carbon, J. (1980), Gene 10: 157-166.), once introduced in the host cell Z. bailii, allow the autoreplication of the expression vector. Any ARS-like sequence, isolated from the yeast genus Zygosaccharomyces, and especially from Z. bailii, or from similar yeasts, can be used. The development of expression vectors containing all or part of the endogenous pSB2 plasmid, along with the expression cassette and a transformation marker, is also possible.

It has been found that the APT gene (Hadfield C., Jordan B.E., Mount R.C. Pretorius G.H.J. and Burak E. (1990) Curr.Genet. 18:303-313), coding the resistance to the antibiotic geneticin (G418) can be used as transformation marker for the selection, propagation and growth of recombinant clones. Other dominant markers are available for yeasts (formaldehyde resistance, phleomycin resistance, fluoroacetate resistance , etc, reviewed in Van den Berg M. and Steensma Y.H. (1997), Yeast 13:551-559), and have good chance to be active for Z. bailii too. Indeed, a description of the use of SFA1 gene, giving formaldehyde resistance, in Z. bailii has been very recently reported (Mollapour M. and Piper P.W, supra).

Other auxotrophic markers from other yeasts as well as from Z. bailii can be successfully also used. In fact, the rescue of auxotrophic deficiency of S. cerevisiae (ura3, his3, trp1 and leu2) with sequences isolated from Z. bailii, probably coding for homologous gene products, has been shown. It is therefore likely that those auxotrophic markers can be "shuttled" between said yeasts (Rodrigues et al., supra; Mollapour M. and Piper P.W, supra).

It has finally been found that the stability of the expression vector is strongly increased, even in absence of the selective pressure, for the presence of CEN sequences, and in particular the sequence CEN4 (Mann C. and Davis R.W. (1986) Mol Cell Biol, 6:241-245) from S. cerevisiae. These sequences drive the correct repartition of the plasmids during the cellular division. It follows that other CEN sequences, either homologous or derived from S. cerevisiae or other organisms, can also be successfully used.

Vectors may also bear sequences ensuring the propagation in Escherichia coli and/or in S. cerevisiae.

The transformation protocol developed avoids the enzymatic digestion of the cell wall, a common procedure during the transformation of the Zygosaccharomyces genus. The protocol utilises an electroporation procedure similar to those already published for S. cerevisiae and Kluyveromyces lactis. Transformation efficiencies can be increased with a treatment based upon monovalent cations, as Lithium salts, and/or reducing agents, as dithiothreitol or β-mercaptoethanol, and/or with the addition of nucleic acid as carrier

A further increase of the transformation efficiency is obtained with the incubation of the treated cells in a regenerative medium. The transformation method is carefully described in the Example n.3.

Said procedure can be applied to all the yeasts of the species Z. bailii, preferably to the strains ATTC36947, ATTC60483 and ISA 1307.

According to the present invention, any gene, either prokaryotic as well as eukariotic, homologous or heterologous, can be introduced and expressed in Z. bailii. The secretion of the recombinant gene product in the culture medium can be obtained by cloning suitable DNA sequences.

This invention can be used for the large scale production of polypeptides having an industrial interest, or giving the transformed strain an industrial interest.

Some examples of proteins that can be produced according to the

invention comprise: interferons, growth hormone, Tissue Plasminogen Activator, surface antigen of hepatitis B virus, erythropoietin, interleukins (ILs), colony stimulating factors (CSFs), nerve growth factor (NGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), Tumor Necrosis Factor (TNF), urokinase, clotting factors (XIIIa), β-endorphin, antibodies, α-galactosidase, endochitinase, esoglucanase, lysozime, tetanus toxin, α-amylase, glucoamylase, prochymosin, uridine, xylose isomerase etc.

The choice of a particular Z. bailii host strain, of the genetic markers and of the regulating sequences may depend on the different product. It has been found that expression of heterologous proteins can be preferably obtained using either a constitutive (ADH1) as well as an inducible promoter (GAL_{1-10}/CYC1) of S. cerevisiae (Porro D., Martegani E., Ranzi B.M. and Alberghina L. (1991) Appl.Microbiol.Biotechnol. 34:632-636). Other promoters from S. cerevisiae, from other «non-conventional» yeasts as well as from Z. bailii, can also be used for the production of heterologous proteins.

Examples of suitable promoters from S. cerevisiae include: TPI, PGK, GAP, GAL1, ADH1, PHO5, CUP1, Mfα1, or the hybrid promoters GAL/CYC1, GAP/GAL, PGK/GAL, GAP/ADH2, GAP/PHO5, CYC1/GRE. The preferred promoter is the ADH1 promoter from S. cerevisiae .

The transformation and growth conditions may vary depending on the particular vector used and/or of the particular gene expressed.

Brief Description of the Figures

FIG. 1A. Expression vector: pZYGO1.

FIG.1B. Schematic representation of the steps necessary for the construction of pZBlacZ plasmid. (for sake of simplicity, presence of the terminator sequence of ADH1 gene, that is present downstream its promoter is omitted).

FIG.1C. Diagram of pZBlacZ plasmid.

FIG.2. β-galactosidase production in the Z. bailii ATTC60483 strain during the growth in synthetic medium (YNB w/o aminoacids 13 g/l, glucose 50 g/l, G418 200 mg/l). Similar results have been obtained using the Z. bailii strains ATTC36947 and ISA1307. Similar results have been also obtained using, instead of the ADH1 promoter, the inducible hybrid promoter $UAS_{GAL1-10}$/CYC1 of S. cerevisiae (data not shown).

(●) Cells/ml; (Δ) glucose, g/l; (■)β-galactosidase, U/mg of total cell proteins

The invention will be described in more detail by means of the following examples, wherein the expression of β-galactosidase gene is disclosed.

This experimental model, based on the expression of a well known reporter gene, is of course predictive for the expression of any other gene having industrial interest.

EXAMPLE 1

Construction of pZygo1 plasmid, a general expression vector for Zygosaccharomyces bailii.

Plasmid pZygo1 is a multi-purpose expression vector, and it contains all the sequences required to self-replicate in three different hosts: Escherichia coli, Saccharomyces cerevisiae and Zygosaccharomyces bailii. In fact, once provided with the appropriate expression cassette i.e.: promoter – homologous or heterologous gene – terminator, the same plasmid can be used to assay different level(s) of expression in Zygosaccharomyces bailii, Saccharomyces cerevisiae and in Escherichia coli.

Selection of transformation can be achieved either with the dominant marker APT (S. cerevisiae, Z. bailii), either with auxotrophic markers (see «Description of the Invention»).

The APT gene, enclosed in a EcoRV/SmaI fragment from plasmid pFA6-KanMX4 (Wach A., Brachat A., Pohlmann R. and Philippsen P. (1994)

Yeast 10:1793-1808) was subcloned in pBluescript KS+ plasmid (Stratagene) at EcoRV restriction site. APT gene confers geneticin (G418) resistance.

Further, the gene was excised with a SalI/EcoRI digestion and inserted at the corresponding sites of YCplac33 centromeric vector (Gietz D.R. and Sugino A. (1988) Gene 74:527-534), thus yielding pZygo1. Some unique restriction sites are available along the vector, allowing the insertion of the desired expression cassette (Fig.1A)

EXAMPLE 2

Construction of the pZBlacZ plasmid, containing ADH1 promoters from S. cerevisiae and the reporter gene.

First, vector pKAN has been constructed, with the insertion of APT gene, enclosed in a EcoRI/SalI fragment from plasmid pFA6-KanMX4 (Wach A., Brachat A., Pohlmann R. and Philippsen P. (1994) Yeast 10:1793-1808) at EcoRI e SalI restriction sites of YCplac33 centromeric vector (Gietz D.R. and Sugino A. (1988) Gene 74:527-534).

LacZ gene from Escherichia coli has been obtained in the following way. A first digestion with NcoI of the plasmid pLA41(Porro D., Martegani E., Ranzi B.M. and Alberghina L. (1991) Appl.Microbiol.Biotechnol. 34:632-636) was followed by a treatment with the «Klenow fragment» of the enzyme DNA polymerase, in order to obtain blunt end molecules. Those molecules were subsequently digested with BamHI, and a fragment of about 3600 base pairs, containing the coding sequence, was isolated and ligated to the vector pVT102-U (Vernet T., Dignard D. and Thomas D.Y. (1987) Gene 52:225-233), previously treated described hereinbelow. Vector pVT102-U was digested with XbaI, treated with the «Klenow fragment» and further digested with BamHI. The resulting plasmid, named pADH-Z, bears LacZ gene under the control of promoter and terminator of ADH1 gene from Saccharomyces cerevisiae.

Finally, the whole expression cassette (ADH1 promoter - LacZ gene -

ADH1 terminator) was excised from pADH-Z with a SphI digestion, and inserted in to the pKan vector, previously linearized with SphI. The resulting expression plasmid has been named pZBlacZ (Fig.2B-C)

EXAMPLE 3

Transformation of Zygosaccharomyces bailii yeasts with pZygo1 and pZBlacZ.

Z. bailii cells from strains ATTC36947, ATTC60483 and ISA1307 were grown in rich medium YEPD (Yeast Extract 10 g/l, Peptone 20 g/l, Glucose 20 g/l) till a concentration of about $2x10^8$ cells/ml. Cells are collected and incubated for one hour at room temperature, at a density of $1x10^9$ cells/ml, in a freshly prepared solution of 0.1M lithium acetate, 10 mM dithiothreitol, 10 mM Tris-HCl, pH 7.5. Cells are washed once with water and twice with sorbitol 1M, and concentrated to a density of $1x10^{10}$ cells/ml in sorbitol 1M. Electroporation (1.5 kV, 7.5 KV/cm, 25 μF, 200 Ω: GenePulser, Biorad, Hercules, Ca) take place in the presence of 3-5 μg of plasmid.

Cells are recovered with an incubation of 18 hours in 5 ml of YEPD, Sorbitol 1M at 28°. The selection of transformant is obtained by plating the cellular suspension in 20 g/l glucose, 20 g/l Peptone, 10 g/l Yeast Extract, 20 g/l agar, 200 mg/l G418 (Gibco BRL, cat. 11811-031)). Single clones appear in 2-3 days at 28°.

EXAMPLE 4

Heterologous protein production during a batch fermentation of Zygosaccharomyces bailii [pZBlacZ] transformed cells.

Some single clones obtained with the transformation of ATTC60483 strains with pZBlacZ plasmid depicted above, were assayed during a batch growth in synthetic medium (Yeast Nitrogen Base w/o aminoacids 13 g/l, glucose 50 g/l, G418 200 mg/l). Cells were pre-inoculated in the same medium of the experiment, and inoculated in shake flasks (300 ml),

containing 100 ml of fresh medium. Flasks were incubated in shaking bath at 30°C, 200 rpm, and the fermentation was regularly monitored. Cell number was determined with an electronic coulter (Coulter counter ZBI, Porro D., Martegani E., Tura A., Ranzi B.M., (1991) Res. Microbiol., 142 :535-539), after the elimination of eventually cellular aggregates with a sonication step (10 seconds, sonicator Fisher 300, power 35%).

β-galactosidase activity has been assayed as follows. About 10^8 cells are collected, washed with water and resuspended in 300 µl of Z-buffer: $Na_2HPO_4.2H_2O$ (0.06M), $NaH_2PO_4.H_2O$ (0.04M), KCl (0.01M), $MgSO_4.7H_2O$ (0.001M), β-mercaptoethanol (0.05M), pH 7. The cells are added of the same volume of beads Glasperlen (SIGMA) (0.45-0.55 mm), and broken with three cycles: 1' on vortex – 1' ice. Cellular debris are eliminated with a centrifugation at 12.000 rpm, 10'. The supernatant is collected, and it constitutes the total protein extraction. β-galactosidase activity is assayed with 10 µl of extract plus 390 µl Z-buffer, 100 µl ONPG (o-Nitrophenyl beta-D-galactopyranoside) 4 mg/ml, at 30 C. Reaction is stopped with 500 µl of Na_2CO_3 1 M, pH 11.

The protein concentration in the total extract is determined with Kit BIO-RAD Protein Assay (cat. 500-0001).

The enzymatic activity is calculated as:

$$U/ml = (\Delta E_{420}/min \times V.F.) \times (\varepsilon \times d \times V.s.)^{-1}$$

where:

ε: molar extinction coefficient (0.0045 M^{-1} cm^{-1})

d: optical path (1 cm)

V.F.: final volume (ml)

V.s.: sample volume assayed (ml)

1 Unit is defined as the amount of enzyme that produces 1 nmol of o-nitrophenol /min at the temperature of 30° C. Specific activity corresponds to 300.000 Units/mg.

No β-galactosidase activity has been detected in the control strains, i.e. Zygosaccharomyces strains not transformed or transformed with pZygo1.

EXAMPLE 5

Determination of the stability of plasmids pZygo1 and pZBlacZ in cells grown in rich medium, with or without selective pressure.

ATTC60483 cells transformed with pZygo1 or pZBlacZ have been grown in rich media YEPD (Yeast Extract 10 g/l, Peptone 20 g/l, Glucose 20 g/l, G418 200 mg/l) till their stationary phase.

After the determination of the percentage of the population retaining the plasmid, 95 ± 2 % (see ahead), cells were transferred in fresh medium, without selective pression (i.e. YEPD without G418), and were grown for 15 generations. Finally, it was evaluated the percentage of cells still retaining the plasmid. In such a way a low rate of plasmid loss per generation (16 ± 2 % per generation) was calculated for both plasmids.

Determination of the percentage of population retaining the plasmid.

About 500 cells are transferred, after a sonication step (see above) onto YEPD plates, without any selection. In these conditions, each viable cell originates a clonal colony. Once the colonies have emerged, them are stricked onto a YEPD plate containing G418: only cells retaining the plasmid are able to grow. The percentage of cells having the plasmid in the original population is calculated as the ratio between the number of colonies in the two plates.

CLAIMS

1. A process for the expression of homologous and heterologous proteins by culturing Zygosaccharomyces bailii strains transformed with expression vectors having a gene coding for such as protein.

2. Expression vector for Z. bailii strains containing ARS sequences from S. cerevisiae, homologous CEN (centromeric) sequences or derived from S. cerevisiae, a gene coding for a protein, a promoter controlling the expression and a marker.

3. A vector according to claim 2 in which the ARS sequence is the ARS1 sequence.

4. A vector according to claim 2 or 3 where the centromeric sequence is CEN4 from S. cerevisiae.

5. A vector according to anyone of the claims 2,3,4 derived from the plasmid pSB2 of Z. bailii.

6. A vector according to claims 2,3,4,5 where the marker is the gene for resistance to geneticin

7. A vector according to claims 2-6 where the promoter is ADH1 and/or GAL_{1-10}/CYC1 from S. cerevisiae.

8. A strain of Z. bailii transformed with a vector from claims 2-7.

9. A strain according to claim 8 derived from ATCC36947, ATCC60483 or ISA1307 strains.

10. A method of transformation of Z. bailii strains including electroporation in the presence of monovalent ions, reducing agents and/or nucleic acids.

FIGURE 1-A

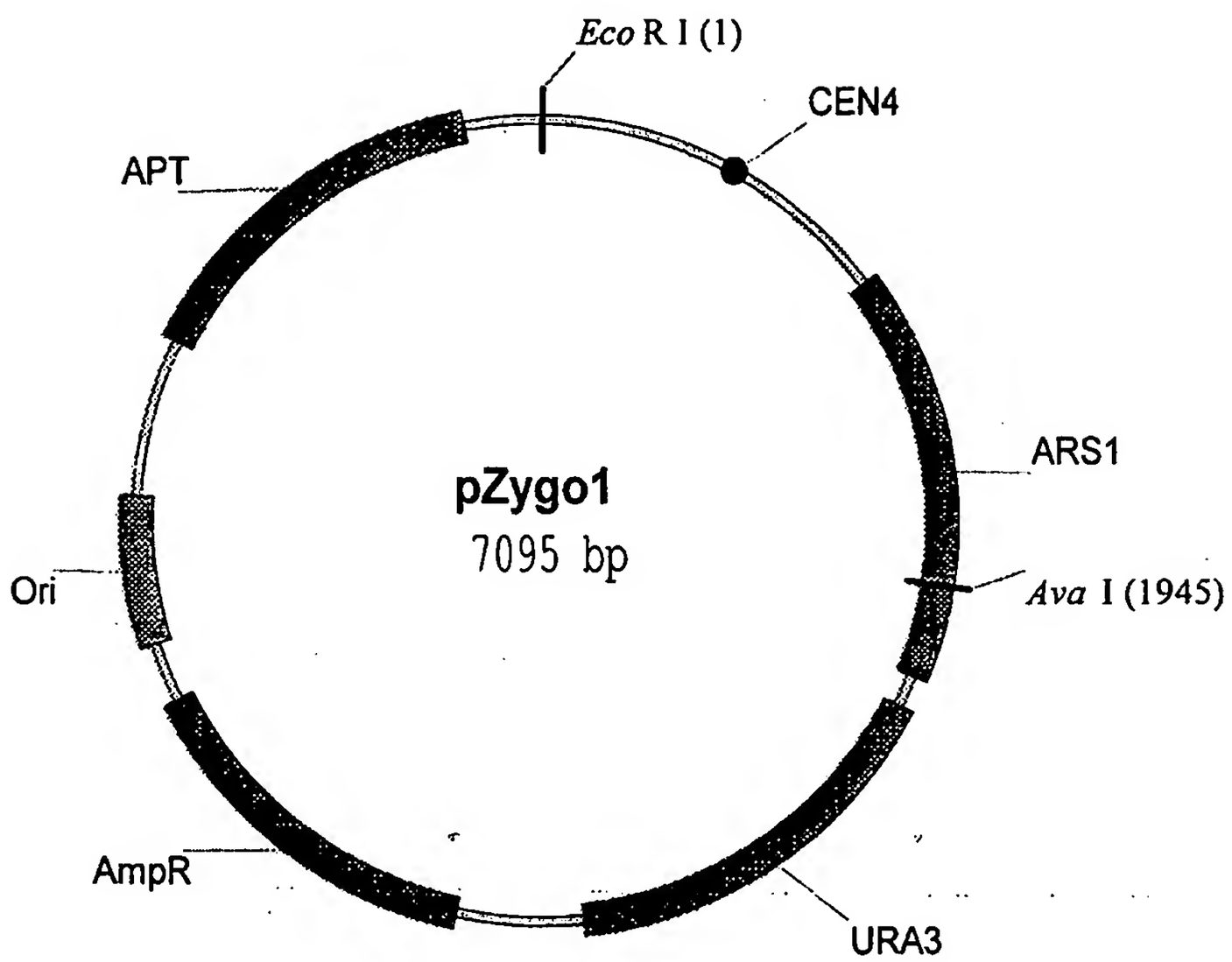


FIGURE 1-B

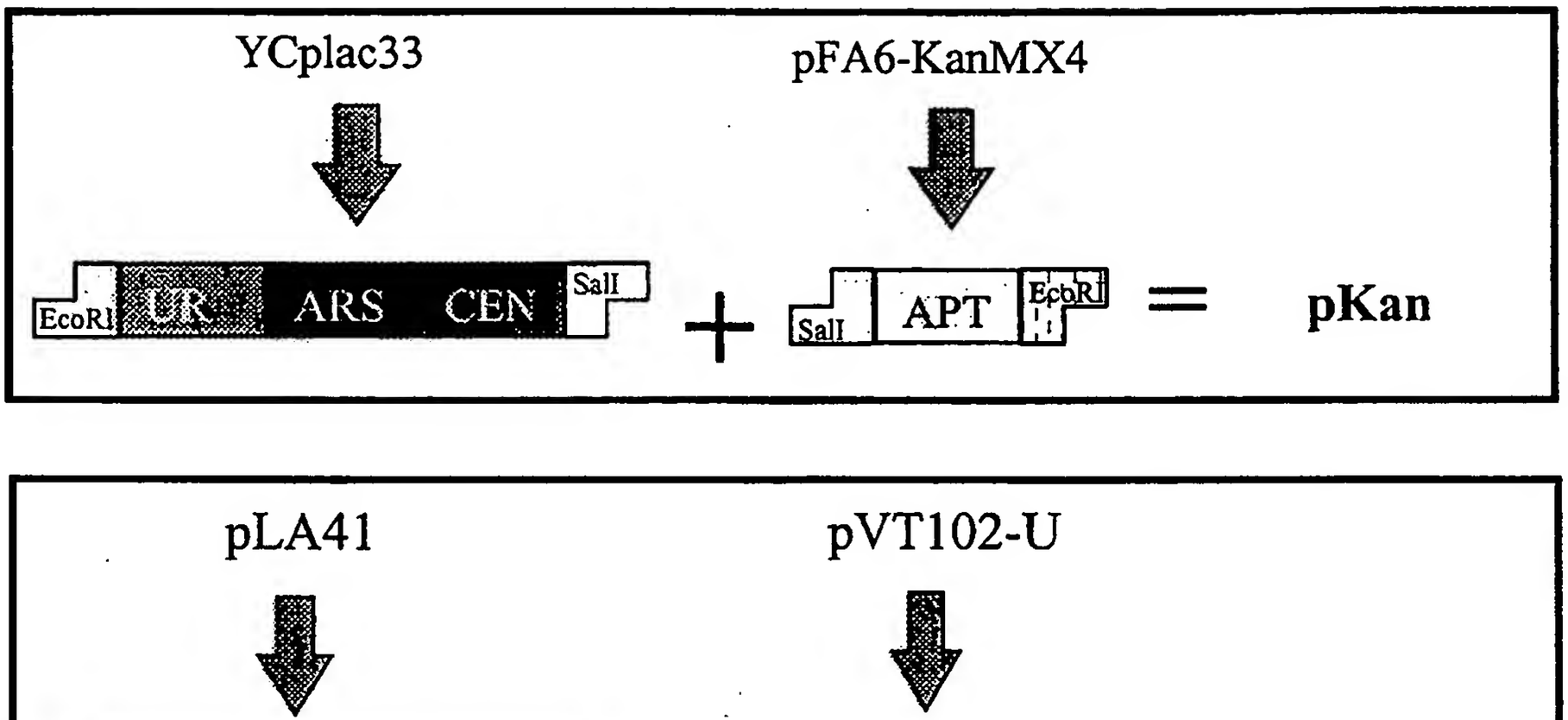

YCplac33
pFA6-KanMX4
EcoRI
URA3
ARS
CEN
SalI
SalI
APT
EcoRI
pKan
pLA41
pVT102-U
NcoI
LacZ
NcoI
XbaI
XbaI
BamHI
LacZ
BamHI
pADH-Z
pKan
pADH-Z
SphI
URA3
ARS
CEN
APT
SphI
SphI
ADH1
LacZ
SphI
pZBLac

FIGURE 1-C

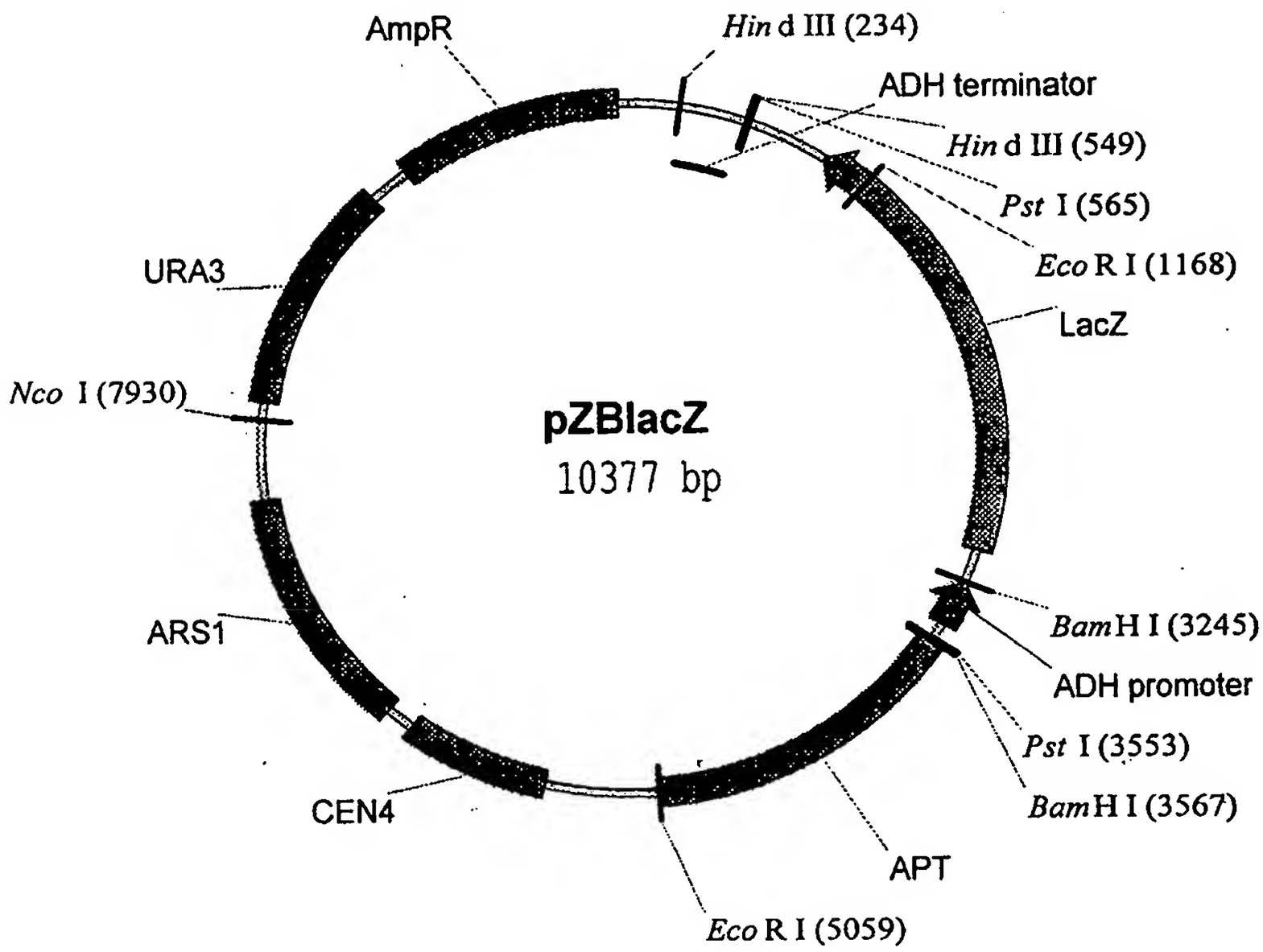
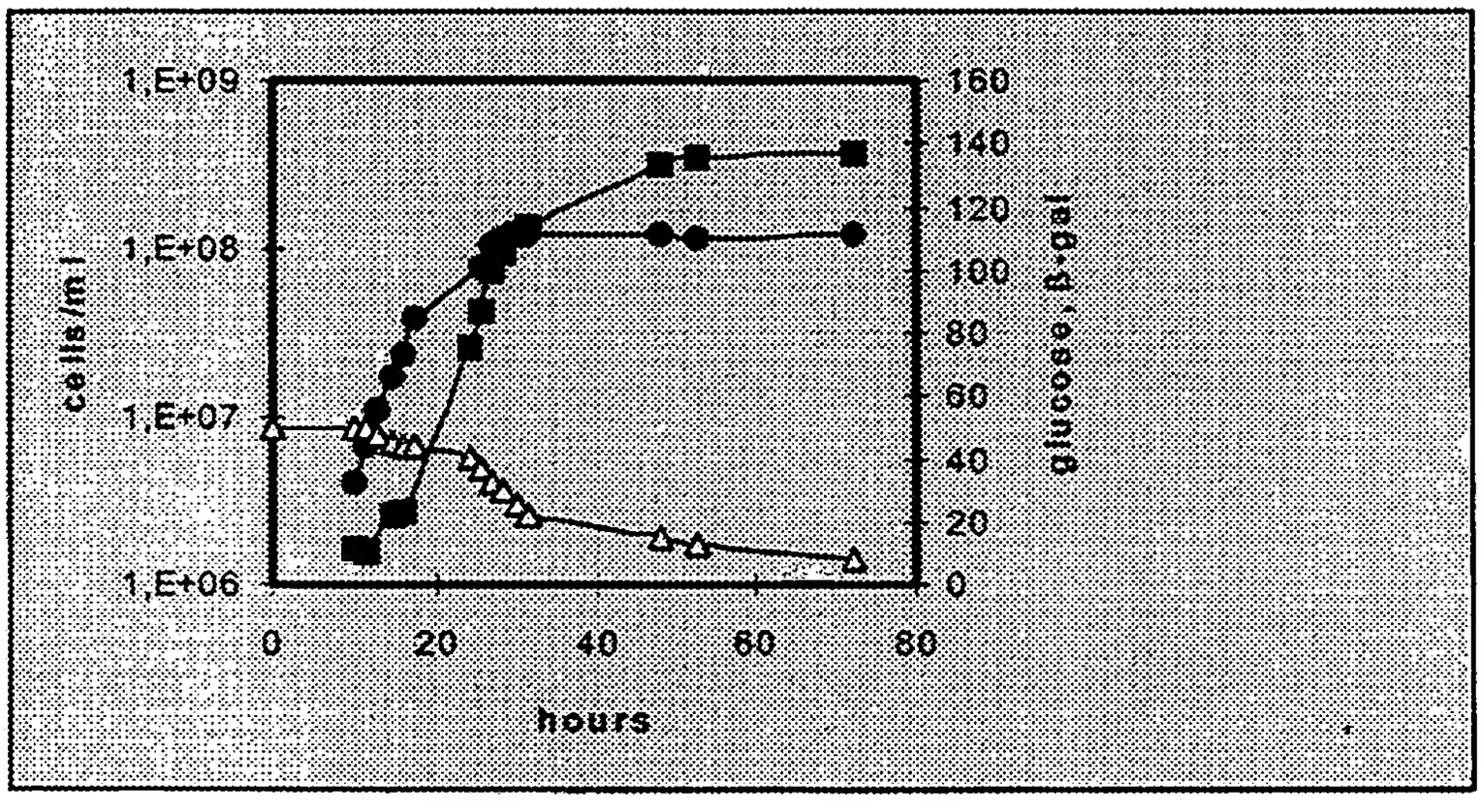

AmpR
Hin d III (234)
ADH terminator
Hin d III (549)
Pst I (565)
Eco R I (1168)
LacZ
URA3
Nco I (7930)
pZBlacZ
10377 bp
ARS1
BamH I (3245)
ADH promoter
Pst I (3553)
BamH I (3567)
CEN4
APT
Eco R I (5059)

FIGURE 2


1,E+09
1,E+08
1,E+07
1,E+06
cells/ml
0
20
40
60
80
hours
160
140
120
100
80
60
40
20
0
glucose, g/l